Investigation of biofilm resistance to antimicrobial agents
by Katherine Jean Grobe

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The goal of this project was to investigate aspects of biofilm resistance to antimicrobial agents using a novel artificial biofilm system. Experiments were done with four different antimicrobial agents (chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound, QAC) in an effort to explain the increased resistance to antimicrobial agents of biofilms over their planktonic counterparts. Data was collected as survival vs. time for each agent. The two mechanisms of resistance considered were 1) transport: a failure of the biocide to fully penetrate the biofilm and 2) physiology: an inherent physiological heterogeneity within the biofilm. A simple disinfection model was utilized to discern the concentration dependence of biofilm disinfection.

Bacteria in biofilms were clearly less susceptible to all biocides than were the same microorganisms when grown in a conventional suspension culture. For example, using 50 mg/L glutaraldehyde it took approximately 20 minutes to achieve a 2 log reduction in viable cell numbers in planktonic experiments but almost 600 minutes to achieve this same level of killing in the biofilm. In general, the susceptibility of bacteria in biofilms was reduced by approximately an order of magnitude compared to planktonic bacteria. Biofilm results indicated that both chlorine and glutaraldehyde penetration into the biofilms was retarded and that poor penetration likely contributed to reduced biofilm susceptibility. It was also shown that in biofilm disinfection by these two agents there is a higher dependence on concentration than in a planktonic suspension. It is more advantageous to dose a biofilm with a high concentration of these agents for a short period of time rather than a low concentration for a proportionately longer time. On the other hand results from biofilm disinfection by ciprofloxacin and QAC were biphasic and consistent with the existence of an inherently resistant subpopulation within the biofilm. The demonstration of unambiguous biofilm resistance to biocides of various chemical natures highlights the need to continue to employ biofilm testing methodologies in designing and optimizing applications of these agents. The insights obtained in this investigation suggest possible approaches for improving biofilm control.

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APPROVAL

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Katherine Jean Grobe

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The goal of this project was to investigate aspects of biofilm resistance to antimicrobial agents using a novel artificial biofilm system. Experiments were done with four different antimicrobial agents (chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound, QAC) in an effort to explain the increased resistance to antimicrobial agents of biofilms over their planktonic counterparts. Data was collected as survival vs. time for each agent. The two mechanisms of resistance considered were 1) transport: a failure of the biocide to fully penetrate the biofilm and 2) physiology: an inherent physiological heterogeneity within the biofilm. A simple disinfection model was utilized to discern the concentration dependence of biofilm disinfection.

Bacteria in biofilms were clearly less susceptible to all biocides than were the same microorganisms when grown in a conventional suspension culture. For example, using 50 mg/L glutaraldehyde it took approximately 20 minutes to achieve a 2 log reduction in viable cell numbers in planktonic experiments but almost 600 minutes to achieve this same level of killing in the biofilm. In general, the susceptibility of bacteria in biofilms was reduced by approximately an order of magnitude compared to planktonic bacteria. Biofilm results indicated that both chlorine and glutaraldehyde penetration into the biofilms was retarded and that poor penetration likely contributed to reduced biofilm susceptibility. It was also shown that in biofilm disinfection by these two agents there is a higher dependence on concentration than in a planktonic suspension. It is more advantageous to dose a biofilm with a high concentration of these agents for a short period of time rather than a low concentration for a proportionately longer time. On the other hand results from biofilm disinfection by ciprofloxacin and QAC were biphasic and consistent with the existence of an inherently resistant subpopulation within the biofilm. The demonstration of unambiguous biofilm resistance to biocides of various chemical natures highlights the need to continue to employ biofilm testing methodologies in designing and optimizing applications of these agents. The insights obtained in this investigation suggest possible approaches for improving biofilm control.

BACKGROUND

Biofilms and Biofilm Resistance

Biofilms cause myriad problems. For example, they are responsible for inefficiency in cooling towers, souring of oil fields, and clogging of drains. Many treatment applications have been developed in industry for the control of biofilms. In order to expedite transfer of effective treatments into the marketplace, a method is needed to allow biofilm resistance and resistance mechanisms to be investigated. This project investigates the utility of an artificial biofilm system by testing it against four diverse antimicrobial agents. Proposed mechanisms of resistance are examined and transport properties of the artificial biofilm system studied.

Microorganisms in biofilms are almost always found to be profoundly less susceptible to antimicrobial agents than are their freely suspended counterparts. Examples of biofilm resistance to glutaraldehyde, for example, can be found in the literature (Cheung and Beech 1998; Dewar 1986; Eagar, et al. 1998; Grab and Theis 1993; Green and Pirrie 1993; Leder 1989; McCoy, et al. 1986; Reinsel, et al. 1996; Ruseska, et al. 1982; Stewart, et al. 1998; Duguid, et al. 1992; Vess, et al. 1993; Whitham and Gilbert 1993). Because biofilms exhibit distinct physiologies and physical

properties, the conclusions drawn from antimicrobial experiments performed with planktonic (freely suspended) microorganisms may be inappropriate for biofilm applications. For example, in a comparison of two forms of chlorine, hypochlorous acid and monochloramine, it has been shown that monochloramine, which is a weaker disinfectant when tested against planktonic microorganisms, was actually the superior agent when tested against biofilms (Stewart, et al. 1999). This result underscores the importance of performing biocide efficacy tests against biofilms.

The Antimicrobial Agents Investigated

Chlorine

Chlorine has diverse applications. It is used in drinking water distribution systems, pools and spas, waste water treatment facilities, in-plant disinfection, and many common household cleaners. Its method of antimicrobial action is unknown, but some hypothesize that chlorine inhibits certain enzymes preventing particular metabolic reactions to take place (Fair et al. 1948; Dychdala 1991). The biocidal action of chlorine is dependent on a number of factors including pH and temperature. Depending on pH, two different forms of chlorine will prevail. Sodium hypochlorite will disassociate into chlorite and hypochlorite ions. It is the hypochlorite ion that is the more effective antimicrobial agent (Dychdala 1991). Chlorine treatment values typically range from 1 ppm in drinking water treatment to upwards of 20 ppm in industry.

Glutaraldehyde

In many of the application areas of glutaraldehyde, including oilfield control of souring and corrosion, industrial water treatment, and sterilization of medical instruments, biofilms are known to be present and probably represent the major target for microbial control. Glutaraldehyde was first investigated as an antimicrobial agent as an alternative to formaldehyde as a fixative. It is widely used as a chemisterilizer for medical instruments that can not be sterilized by traditional heat and pressure methods. Unhindered by organic matter, it is effective against bacteria, fungi, viruses and spores. Its action is dependent on pH, temperature and concentration. Glutaraldehyde is commonly used in the oil field for the treatment of sulfate reducing bacteria (Scott and Gorman 1991).

Ciprofloxacin

Ciprofloxacin is an antibiotic frequently used to treat infections. It is a fluoroquinolone molecule that targets DNA gyrase, a maintenance enzyme responsible for the superhelical twists of DNA (Wolfson and Hooper 1985). It is these super helical twists that regulate the binding of proteins to DNA. DNA gyrase is required for DNA replication, transcription, repair and recombination. The exact mechanism of ciprofloxacin as an antibiotic is unknown, but it is hypothesized that DNA gyrase cleaves chromosomal DNA, thereby blocking DNA replication (Wolfson and Hooper 1985). The MIC value for the bacterial strain used in this study, ERC1, was reported to be 0.25

μg/mL (Vrany, et al. 1997). There is conflicting evidence as to whether ciprofloxacin is a growth-rate dependent antibiotic. Some speculate the susceptibility of bacteria when challenged with ciprofloxacin increases with the specific growth-rate (Duguid, et al. 1992). An earlier study showed that ciprofloxacin is not transport limited in a thin *P. aeruginosa* biofilm (Vrany, et al. 1997; Anderl, et al. 1999).

Quaternary ammonium compound

Quaternary ammonium compounds (QACs) are used in slightly different applications than the aforementioned antimicrobial agents. QACs are the active ingredients in textile chemicals, oilfield chemicals, polyurethane foam catalysis and epoxy curing agents (Albemarle Corporation 1999). A surfactant long considered a biocide, QACs adsorb to the cell wall, diffuse within and bind to the cytoplasmic membrane. Once they have disrupted the membrane, it is the release of potassium ions and cell constituents that cause cell lysis. Not a very effective chemisterilizer for medical instruments, the cationic structure has been proven effective against bacteria in fabrics (which have an inherent negative charge), thus are used in detergents. QACs are also used to treat algae in swimming pools. They are also used frequently to sterilize food contact surfaces such as utensils, pasteurizing equipment and other food synthesis machinery (Merianos 1991).

Artificial Biofilms

An expedient and repeatable method of testing antimicrobial agents is needed to characterize the nature and extent of biofilm resistance to develop more practical approaches to biofilm control. Researchers at the Center for Biofilm Engineering (CBE) have devised a simple, repeatable artificial biofilm test system for characterizing the efficacy of antimicrobial agents against biofilms; alginate gel bead biofilms challenged by antimicrobial agents and sampled over time allow insight into the mechanisms of resistance displayed by biofilms.

Alginate has been used as a matrix for cell immobilization in medicinal and industrial applications. It has been used for the production of ethanol by yeast, mass production of artificial plant seeds and drug and enzyme immobilization (Smidsrod and Skjak-Braek 1990). The use of alginate and agar as a simulated matrix for artificial biofilms has been a recent development in the literature (Stewart, et al. 1998; Coquet, et al. 1998; Jouenne, et al 1994). The alginate matrix is highly hydrated and diffusion readily takes place, simulating the polymer matrix in naturally occurring biofilms (Smidsrod and Skjak-Braek 1990). Such immobilized cells are more resistant to killing than are planktonic cells (Whitham and Gilbert 1993; Keweloh, et al. 1989; Coquet, and others 1998). A particularly interesting extension of this idea is the use of implanted agar gel beads in rat lungs in an effort to model cystic fibrosis disease. The colonization of *P. aeruginosa* in the lungs of severely ill patients has recently been considered a biofilm disease (Costerton, et al. 1999). Researchers have shown that these implanted beads

effectively model the chronic pulmonary disease in rats, allowing the virulence of different strains of *P. aeruginosa* to be studied (Woods, and others 1982).

Mechanisms of Biofilm Resistance

One of the proposed mechanisms of biofilm resistance is a failure of the biocide to penetrate the biofilm. Reactive oxidants like chlorine and hydrogen peroxide are often ineffective biocides against biofilms. Transport limitation may be one explanation for their ineffectiveness. The neutralizing capacity of the components of the biofilm makes penetration of the biocide difficult. Penetration failure can be explained in two different ways. In the first model, the agent reacts stoichiometrically with biomass continually consuming the neutralizing capacity of the biofilm and penetrating deeper. In this model, the biocide must deplete all of the neutralizing capacity of the biofilm before it can penetrate fully. In concept, there are a concrete number of reactive sites within the biofilm and the agent must react with each site before it can proceed within the biofilm. The second model of penetration failure is a specific catalytic reaction within the biofilm, in which reaction-diffusion equilibrium occurs. This model suggests that the biocide only reacts with the biotic portion of the biofilm. The penetration would only be limited if the biofilm responds with an enzymatic process (Dodds, et al. 1999).

Other biofilm research has indicated that there is an inherent physiological heterogeneity within the biofilm (Huang, et al. 1998). A sensitive fraction of cells may be easily killed off by the antimicrobial agent, while a resistant fraction remains harder to

kill. Evidence for this physiological resistance mechanism explains why some antibiotics readily diffuse into biofilm, yet kill it incompletely (Vrany, et al. 1997; Anderl, et al. 1999). An observed limited zone of protein synthesis activity in biofilms also supports this mechanism hypothesis (Brown and Gilbert 1993). Cells that are not undergoing protein synthesis or growth are known to be more resistant than actively growing cells. Research at the CBE has demonstrated that there is spatial heterogeneity related to alkaline phosphatase expression, which in turn is related to starvation of the biofilm cells. It has been shown that starved cells are more difficult to kill, suggesting a less susceptible "biofilm-phenotype" (Huang, et al. 1998). Nutrient limited and slow growing, starved cells have been shown to exhibit an increased resistance to antimicrobial agents (Duguid, et al. 1992; Brown and Gilbert 1993). While these mechanisms of biofilm resistance likely do not exist independently, it should be possible to discern which mechanism may predominate in biofilm disinfection by a particular agent.

Analysis of survival versus time plots provides insight into the mechanism of resistance shown by biofilms to a particular antimicrobial agent (Dodds, et al. 1999; Figure 1). A concave down curve is characteristic of a transport limited biocide (Figure 1). The initial relatively flat portion of the kill curve suggests a period of time where the biofilm is still penetrating the biofilm. Once full penetration has been reached (indicated by the inflection point) the biocide becomes more effective. Concave up curvature suggests that physiology is the dominating mechanism of resistance (Figure 1). The initial decrease in the bi-phasic curve represents the sensitive fraction of cells within the biofilm being killed. The inflection point and subsequent flatter portion of the

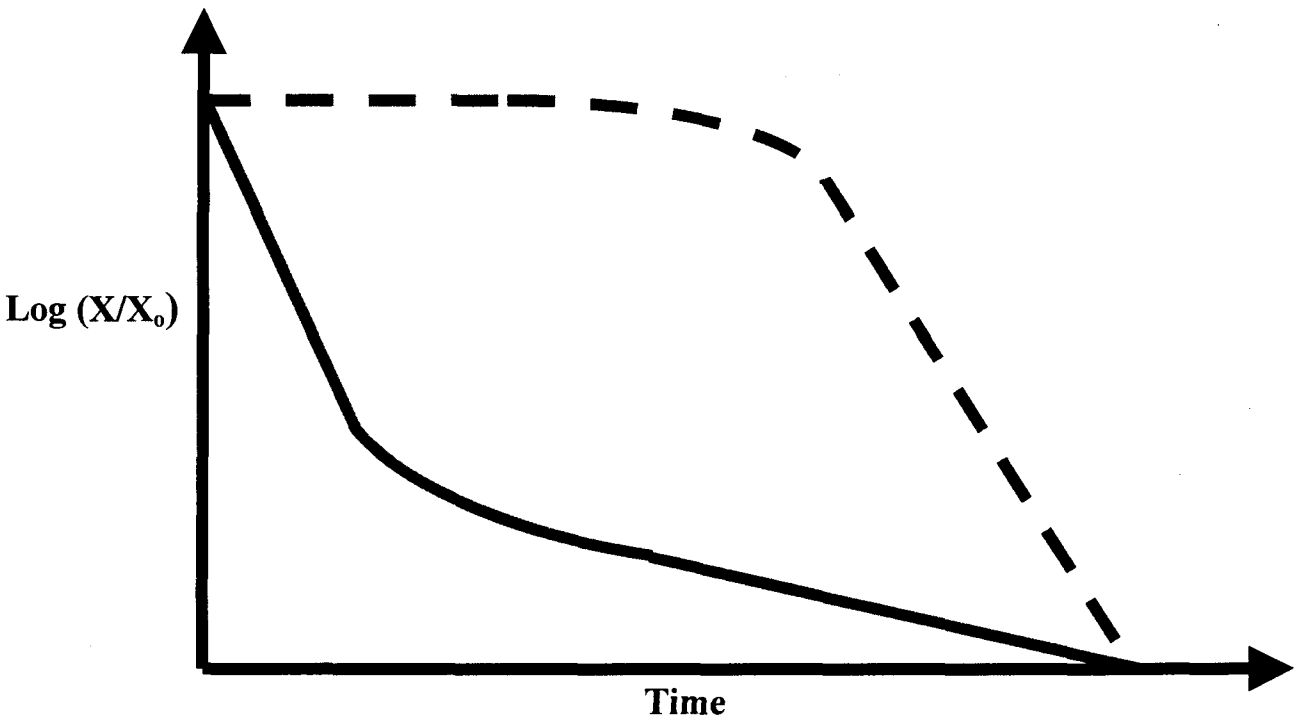

Figure 1. Predicted biofilm disinfection curve shapes (Dodds, et al. 1999). The dashed line represents a transport limitation while the solid line indicates a physiological heterogeneity within the biofilm.

physiological curve indicates a more resistant population that is more difficult to kill. A computer model created by Mike Dodds at the CBE to analyze biofilm disinfection phenomena was used in this analysis (Dodds, et al. 1999).

Traditional lab biofilms are time-consuming and tedious to grow, making collection of adequate survival versus time data difficult. This type of data is rarely found in the literature for biofilms. The artificial biofilm system is attractive because of the large number of consistent biofilm samples that can be created in a manner of minutes.

In this project an artificial biofilm disinfection system was developed to study four antimicrobial agents: chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound. Objectives included analysis of concentration dependence of each particular agent, utilization of a computer model to analyze physiological model disinfection parameters, and analysis of transport properties within the artificial biofilm system.

MATERIALS AND METHODS

Artificial biofilm gel beads were created and used in disinfection experiments varying the concentration of the four biocides tested, chlorine, glutaraldehyde, ciprofloxacin, and QAC. Planktonic experiments were also performed at the same concentration tested with the artificial biofilm system. A simple disinfection model was used to capture the concentration dependence of each particular agent. Transport of chlorine and glutaraldehyde was analyzed using an observable modulus. Lastly, a computer model created at the CBE was used to gain insight into a physiological based mechanism of biofilm disinfection.

Artificial Biofilm Test System

Artificial biofilms were created by entrapping bacteria, in this case *Pseudomonas aeruginosa*, in alginate gel beads. The gel beads were then suspended in a nutrient medium overnight to allow for growth of microorganisms in the beads and adoption of the biofilm phenotype. The end result – dense microcolonies dispersed in a highly hydrated gel matrix – simulated the structure of real biofilms. Alginate gel bead artificial biofilms appear to be a flexible, reproducible experimental system for investigating antimicrobial efficacy (Stewart, et al. 1998; Whitham and Gilbert 1993; Xu, et al. 1996). Gel bead artificial biofilm preparation is described in detail below.

A plate of R_2A agar (Difco) was streaked with a lawn of *P. aeruginosa* (ERC 1) and incubated overnight (36°C). Phosphate buffer (9 mL, pH 7.2) was then added to the agar plate and the cells were gently scraped off the plate using a glass hockey stick. The buffer/bacterial suspension was mixed with an equal volume of alginic acid, (Sigma, sodium salt, from *Macrocystis pyrifera*, 4%, 9 mL) to make a final 2% alginate concentration. Next, the alginate/bacterial slurry was placed in a sterile syringe (30 mL) with an attached needle (22 gauge). A stopper attached to a compressed air tank allowed the syringe to be pressurized. When the air was turned on (20 psi), a stream of small droplets was forced out the needle and dropped into a stirred solution of calcium chloride (50 mM). The calcium cross-linked the alginate and semi-solid beads with entrapped cells were formed. The beads were allowed to stir in the calcium chloride solution (50 mM) for approximately 20 min, then rinsed in a dilute solution of calcium chloride (5 mM). The beads were incubated overnight (36°C) on a rotating shaker in 1/10 strength nutrient broth with added calcium chloride (5 mM) to maintain the bead structure. The mean gel bead diameter (2.4 mm) was measured by lining up 10 beads on a ruler and determining the mean diameter.

Biofilm Disinfection

Bacterial cells in artificial biofilm gel beads were challenged with an antimicrobial agent (chlorine, glutaraldehyde, QAC, and ciprofloxacin) at varying concentrations at room temperature (23°C). The disinfection solution was made from a

stock solution of phosphate buffer (containing 0.085g/L potassium dihydrogen phosphate and 0.4055 g/L magnesium chloride) with added calcium chloride (5 mM) to support the bead structure. The desired amount of antimicrobial agent was then added to the phosphate buffer and the concentration verified when possible. At the start of the experiment, approximately 250-300 beads were placed in the magnetically stirred disinfection solution (500-700 mL) and beads (10) were removed at various time points. The sampled beads were placed in 5 mL of a solution containing a neutralizing agent (Table 1) and sodium citrate (50 mM). The sodium citrate dissolves the bead structure.

Table 1. Neutralizing agents for the antimicrobial agents investigated.

Agent	Neutralizer
Chlorine	Sodium Thiosulfate (50 mM)
Glutaraldehyde	Glycine (1%)
Quaternary Ammonium Compound	Tryptic Soy Broth
Ciprofloxcin	None; dilution

The bead-citrate solution was refrigerated for two hours while the beads dissolved, then diluted and plated out on R_2A using the drop plate method (Hoben, et al 1948; Reed, et al. 1948). The plates were incubated overnight (36°C) and counted. A control experiment was conducted in the same manner, with the disinfection solution replaced by phosphate buffer (pH 7.4). Number of experimental replicates is tabulated in Table 2.

Planktonic Cell Disinfection

Planktonic cells were challenged with an antimicrobial agent (chlorine, glutaraldehyde and QAC) at various concentrations at room temperature (23°C). A planktonic culture of *P. aeruginosa* (ERC1) was grown on a rotating shaker (36°C) to mid-log phase in 1/10 strength nutrient broth (Difco) with added calcium chloride (5 mM, for consistency with beads experiments). Aliquots of this culture were centrifuged (7.5 min, 10,000 rpm) and the supernatant broth decanted. The bacterial pellet was resuspended with phosphate buffer (pH 7.4) by vortexing. The disinfection solution (18 mL) was made from a stock solution of the particular antimicrobial agent (chlorine: approximately 50,000 ppm, glutaraldehyde: 250 ppm, QAC: BARQUAT MB80-80% active alkyl dimethyl benzyl ammonium chloride) and phosphate buffer (pH 7.4) so that the final concentration, after the bacterial suspension was added (2 mL), was the desired concentration (the nominal concentration reported). The solution was sampled at various time points (1 mL) and the aliquot was neutralized in the appropriate neutralizer (9 mL), diluted and plated on R_2A agar using the drop plate method. The plates were incubated overnight (36°C) and counted. A control experiment was conducted in the same manner, with the disinfection solution replaced by phosphate buffer (pH 7.4).

The planktonic disinfection by ciprofloxacin was done in a slightly different manner. A planktonic culture of *P. aeruginosa* (ERC1) was grown on a rotating shaker

(36°C) to mid-log phase in 1/10 strength nutrient broth with added calcium chloride (5 mM, for consistency with beads experiments). Aliquots of this culture were centrifuged (7.5 min, 10,000 rpm) and the supernatant broth decanted. The bacterial pellet was resuspended with phosphate buffer (pH 7.4) by vortexing. The disinfection solution (45 mL) was made from a stock solution ciprofloxacin (0.0025 g/mL) and phosphate buffer (pH 7.4) so that the final concentration, after the bacterial suspension was added (5 mL), was the desired concentration (1, 5, or 25 μg/mL). Planktonic disinfection was performed at room temperature (22°C). The solution was sampled at various time points (1 mL) and vortexed (7.5 min, 10,000 rpm), the disinfection solution decanted and the challenged bacterial pellet resuspended in phosphate buffer (pH 7.4). The resuspended solution was again vortexed (7.5 min, 10,000 rpm), the wash solution decanted and the bacterial pellet again resuspended in phosphate buffer. The resuspended, washed bacterial sample was then plated on R_2A agar using the drop plate method and incubated overnight (36°C) and the culturable counts determined. Number of experimental replicates is tabulated in Table 2.

Chlorine Concentration Assay

Chlorine concentration was determined using the DPD colorimetric method (Hach). Aliquots of the chlorine disinfection solution were taken at various time points during each experiment to monitor the residual concentration.

Table 2: Number of experimental replicates for each antimicrobial agent tested. ND denotes not determined.

Antimicrobial Agent (mg/L)	# Planktonic experiment replicates	#Biofilm experiment replicates
Untreated Control:	2	2
Chlorine:		
10	4	3
20	3	3
80	4	2
Glutaraldehyde:		
25	3	2
50	4	2
100	3	3
200	ND	2
250	3	ND
Ciprofloxacin:		
1	3	3
5	7	3
25	3	3
QAC:		
50	4	4
100	3	2
250	4	5
500	ND	2
1000	ND	2

Glutaraldehyde Concentration Assay

Glutaraldehyde concentration was determined by gas chromatography. Samples (1 mL) of the glutaraldehyde disinfection solution were taken at various time points during each experiment to monitor the residual concentration and stored under refrigeration (4°C) in Target silanized vials (Fisher) until they could be injected, which was in no case longer than 24 hours. Gas chromatography analysis was performed on a Hewlett-Packard 5890 Series chromatograph. The operating temperatures were as follows: detector 250°C, injector: 190°C, oven: 185°C. The column head pressure was approximately 38 psi. Working standards were prepared from a stock solution of UCARCIDE 250 (Union Carbide Corporation) and a standard curve was constructed. The syringe (Hamilton P/N 80337) was rinsed with water between injections and several times with the sample before each injection. Working standards were injected approximately every 15 injections. The injections (2 µl) were done in duplicate for each sample.

Transmission Electron Microscopy Sample Preparation

Alginate gel beads with entrapped *P. aeruginosa* were prepared as described above and incubated for 24 hours in 1/10 strength nutrient broth (36°C). The beads were then fixed in glutaraldehyde (2.5%) in phosphate buffer (pH 7.4) with added calcium chloride (5 mM, to support bead structure). The beads were then washed (3x, 15 min) in the phosphate buffer. Next, the beads were stained with osmium tetroxide (1%) in the calcium enhanced phosphate buffer. The beads were again washed (3x, 15 min) in the

calcium enhanced phosphate buffer. The beads then underwent a series of dehydration steps: 50% ethanol, 15 minutes; 70% ethanol, 15 minutes; 1% uranyl acetate/1% phosphotungstic acid (PTA), 1 hour; 95% ethanol, 15 min; 100% ethanol, 15 minutes; 100% ethanol, 15 minutes; 100% ethanol, 15 minutes; 100% ethanol (2 parts): SPURRS (1 part, Ernest F. Fullam, Inc.), 1 hour; 100% ethanol (1 part): SPURRS (1 part), 1 hour; SPURRS epoxy resin, 8 hours or overnight. Then the beads were embedded in size 00BEEM capsules. The epoxy resin was polymerized for 14 hours (70°C) in oven. A thick section of bead was cut and stained with toluidine blue. The sections were cut and examined using a Jeol JEM-100CX electron microscope.

Computer Modeling

The computer model created by Mike Dodds at the CBE was used to determine parameters for data sets suggesting a physiological model of resistance. This model of resistance assumes that cells reside in either a resistant state or a susceptible state. Full penetration of the antimicrobial agent is assumed and it is assumed that the susceptible population is as susceptible to the antimicrobial agent as planktonic cells (Dodds, et al. 1999). The solution to this model is expressed analytically by:

$$\int_0^1 \frac{X}{X_0} \cdot d\zeta = \varepsilon_s \cdot \exp(-\psi) + (1-\varepsilon_s) \cdot \exp(-p \cdot \psi) \qquad (1)$$

where

$\psi \equiv k_{dis} \cdot C_b \cdot t_{dose}$
k_{dis} = disinfection rate constant
C_b = concentration of biocide in the bulk fluid

t_{dose} = total time biocide was applied to the biofilm
X = viable biomass density in biofilm after disinfection
X_0 = viable biomass density in biofilm before disinfection
ζ = dimensionless spatial parameter

The parameters obtained were:

ε_s = sensitive fraction of cells within biofilm
k_{dis} = disinfection rate coefficient
p = measure of the relative susceptibility of the resistant fraction

The resistant fraction, ε_r, was obtained by $\varepsilon_r = 1 - \varepsilon_s$.

Matlab computer software was used to implement the model (Dodds, et al. 1999).

Resistance Factor

A resistance factor was calculated by dividing the time needed to reach a two log reduction in the viable cell numbers in artificial biofilm by the time for a two log reduction of planktonic cell viable numbers in response to the same concentration of antimicrobial agent.

Analysis of Concentration Dependence

To analyze the antimicrobial agent concentration dependence of planktonic and biofilm killing, the Chick-Watson mathematical model of disinfection was assumed (Haas and Karra 1984)

$$\frac{dX}{dt} = -k_{dis} C^n X \qquad (2)$$

where X is the viable density, t is time , k_{dis} is a disinfection rate coefficient, and C is glutaraldehyde concentration. The exponent n on the concentration captures the concentration dependence of killing. Assuming a constant biocide concentration, the solution to this model is found by integration to be

$$\ln\left(\frac{X}{X_o}\right) = -k_{dis}C^n t \tag{3}$$

The apparent rate of disinfection over the interval from zero to two-log reduction was defined as

$$r = \frac{-\ln(0.01)}{t} \tag{4}$$

Each experimental data set was fit to a third order polynomial using an existing regression function in an Excel™ spreadsheet. The time for a two-log reduction was determined from this fit and the apparent disinfection rate calculated from Equation (4). Combining Equations (3) and (4) we obtain

$$\ln(r) = \ln(k_{dis}) + n\ln(C) \tag{5}$$

A plot of $\ln(r)$ versus $\ln(C)$ should yield a straight line with slope n. A least squares linear regression of this type was performed to calculate n.

There has been some speculation regarding the applicability of Chick's law to non-linear data sets (Haas and Heller 1990; Haas 1980; Haas 1988). This law was utilized in this analysis strictly as a means of comparing the concentration dependence of each agent on planktonic and biofilm cells independent of the mechanism of disinfection. The

duration of a two log reduction was chosen to minimize the non-linearity of each data set while still capturing the disinfection phenomena.

A common practice in the application of antimicrobial agents is to assume that doses in which the product of dose concentration and dose duration, C^*t, is the same will yield the same disinfection efficacy (Wichramanayake 1991; Hass et al. 1990). This method predicts plotting the surviving fraction versus the product of the bulk concentration and treatment time should collapse data from experiments using different concentrations on to a single curve. Correlations of $\log(X/X_0)$ versus the product C^*t were performed to test the applicability of the C^*t concept.

Observable Modulus

The relative rates of reaction and diffusion within the gel beads were evaluated by calculating an observable modulus, Φ, where

$$\Phi = \frac{R_{obs} L_f^{\,2}}{D_e C_o} \tag{6}$$

and R_{obs} is the overall antimicrobial disappearance rate

$$R_{obs} = \frac{\Delta C \cdot V_{sol}}{V_{bead} \cdot \Delta t} \tag{7}$$

where for chlorine:

$$\Delta C = (C_i - C_f) + k \cdot C_0 \cdot t \tag{8}$$

and for glutaraldehyde:

$$\Delta C = (C_i - C_f) \tag{9}$$

where:
C_i = initial biocide concentration (mg/L)
C_f = final biocide concentration (mg/L)
Δt = time interval (min)
V_{sol} = volume of disinfection solution (mL)
V_{bead} = volume of beads (cm^3)
L_f = effective biofilm thickness (volume to surface area radio)(radius of bead/3)
D_e = the effective diffusivity of agent in the beads (cm^2/s)
C_o = mean concentration over time interval (mg/L)
k = first order rate constant of chlorine disappearance without beads (min^{-1})

The effective diffusion coefficient, D_e, was calculated by estimating the diffusivity using correlations published in the literature (Westrin and Axelsson 1991). Different ΔC values were used for chlorine and glutaraldehyde because it was determined that chlorine's volatility (unexposed to beads) was responsible for some of the degradation of chlorine and must be accounted for. Glutaraldehyde did not significantly disperse in the absence of gel beads.

Table 3. Values of the D_{aq} (25°C) employed in observable modulus calculations (Stewart, 1999).

Antimicrobial Agent	**D_{aq} (cm^2/s)**
Chlorine	1.4×10^{-5}
Glutaraldehyde	9.3×10^{-6}

RESULTS

Survival versus time data from disinfection experiments using artificial biofilms and planktonic cells of the same organism were analyzed. Concentration dependence of biofilm and planktonic disinfection was examined using a simple mathematical model of disinfection. Resistance factor calculations were used to measure the relative resistance of the artificial biofilm cells. For chlorine and glutaraldehyde, an observable modulus was calculated as a measure of the relative rates of reaction and diffusion within the biofilm.

Artificial Biofilm Structure

Electron microscopy of gel beads revealed a structure of dense microcolonies embedded in a highly hydrated gel matrix (Figure 2). Using the electron micrograph image, the cell volume fraction was estimated to be 0.23 in the region near the bead surface. The polymer volume fraction was taken as 0.02. The value of the relative effective diffusion coefficient in the gel bead, D_e/D_{aq}, accounting for the presence of polymer and cells (Westrin and Axelsson 1991), was 0.624.

The average radius of the beads was 2.42 mm $\pm$ 0.07. A distribution of initial cell